Proc. Nat. Acad. Sci. USA
Vol. 71, No. 4, pp. 1050–1054, April 1974

Purification and Characterization of the Hormone Initiating Sexual Morphogenesis in *Volvox carteri f. nagariensis* Iyengar

(glycoprotein)

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Communicated by Harold C. Bold, November 26, 1973

ABSTRACT **In *Volvox carteri f. nagariensis* male spheroids secrete into the medium a sexual hormone which controls the initiation of the developmental pathway leading to the formation of sexual embryos. In the absence of the hormone asexual embryos are formed. Analysis of the highly purified hormone indicates that it is a glycoprotein of 27,500–30,000 daltons, composed of one peptide chain, which has a typical composition except for a relatively low content of tryptophan. The glycosidic moiety, which accounts for about 45% of the weight of the molecule, consists of pentoses, hexoses, and amino hexoses. In the bioassay the highly purified hormone stimulates 100% formation of sexual embryos at a concentration of 10^{-10} g/liter and 14.4% sexual embryos at 10^{-11} g/liter (3×10^{-16} M). The activity is remarkably thermostable and resistant to different denaturing procedures.**

Although *Volvox* has been known to science since 1700 (1) the potential of the many species for studies in development, as suggested 65 years ago by Powers (2), has only recently been realized through the formulation of methods for cultivating it in the laboratory and for evoking at will its asexual and sexual phases. Cellular differentiation and development in a multicellular organism would appear to be in their simplest form in *Volvox*, for here the cells are arranged in a single layer on the periphery of a spheroid and consist of only two types, somatic and reproductive. The formation of new individuals is through a series of successive cleavages of an asexual reproductive cell, the gonidium. In certain species the morphological differentiation of the reproductive cells can be observed as unequal cleavages at specific stages of the embryonic development, the gonidia, the eggs, and the spermatogenous cells being delimited in the respective asexual, female, and male embryos at predictable positions and times.

The phenomenon of sexual induction in *Volvox* was first described by Darden (3) in a strain of *Volvox aureus*. In this species a substance was secreted by males which, when added to a population of asexual individuals, would result in the formation in the next generation of sexual embryos rather than asexual ones as would have occurred without this additive. Similar phenomena of sexual induction have since been described in a variety of species and forms of the genus (4, 5). In all species studied, the sexual hormone initiates the developmental pathway leading to the formation of a sexual individual rather than an asexual one, but the substance is not sex-specific. In species in which distinctive males and females are produced in separate clones, the same hormone initiates the formation of sexual embryos in both sexes. The hormone is, however, species-specific (6); it is usually produced only in populations of sexual males, but in one species both male and female populations produce active substances which are both self- and cross-inductive (7).

The sexual hormones from the various species which have been investigated are all inactivated by Pronase digestion (8), and so it has been assumed that they are proteinaceous. Ely and Darden (9) have attempted purification of the hormone in *V. aureus*, and though the yield was too small for analysis it was postulated that it might be a glycoprotein.

The present communication summarizes our efforts toward purification and characterization of the sexual hormone produced in male populations of *Volvox carteri f. nagariensis*. The morphology and embryonic development of this species, the methods of cultivation, the bioassay for the detection of the hormone, and accounts of genetic loci affecting the potency of the male as well as the developmental process of all types of embryos have been discussed in earlier papers (5, 6). Therefore the following paragraphs on the organism and its cultivation include only those details necessary for clarity in this communication.

The organism

The strains of *Volvox carteri f. nagariensis* used in this investigation were HK10 female and 69-1b male. The asexual spheroids of both strains are identical in appearance, having a maximum of 5000 small biflagellate somatic cells and 16 large gonidia (asexual reproductive cells). The female spheroid resembles an asexual one in its size and number of somatic cells, but it is distinctive in having 45 or more small dense eggs rather than the 14–16 large gonidia. The male spheroid is said to be dwarf because it has a maximum of only 512 cells, of which there is a 1:1 ratio of somatic to spermatogenous cells.

New individuals are formed by the gonidia of the asexual spheroid undergoing a series of cleavages whose patterns to form the asexual, the male, and the female embryos are distinctive. The pattern of cleavage, i.e., sexual or asexual, depends on the presence or absence of the sexual hormone produced by the males. In the formation of an asexual embryo (in both male and female strains), the gonidia are morphologically differentiated by unequal cleavages of the cells in the anterior half of the embryo at the division of the 32-celled stage; in the female embryo, the morphological differentiation of eggs occurs by unequal cleavages in the anterior two-thirds of the embryo at the division of the 64-celled stage; finally, in the formation of the male embryo, the spermatogenous cells are formed by unequal cleavages of all the cells at the last

divisions in the embryo, thus producing the typical 1:1 ratio of somatic cells to spermatogenous cells.

Culture methods

Both male and female strains were grown in Volvox medium, a very dilute medium designed by Provasoli and Pintner (10), but the medium was adjusted to pH 8 rather than pH 7 as formerly used. Sodium acetate was added to the medium to a concentration of 0.05% at pH 7.5 when males were grown for production of the sexual hormone. Illumination of 12,000 lux intensity on a 16-hr light/8-hr dark cycle and a temperature of 28–30° during the light period (20° during the dark) resulted in a generation time of 48 hr, and thereby a population increase by a factor of 15, since there are 14–16 asexual reproductive cells in each spheroid. Large populations were grown in half-filled 500-ml or 2000-ml Erlenmeyer flasks through which sterile air was bubbled.

Sexual spheroids appear spontaneously in the male strain at an approximate frequency of 1 in every 20,000 embryos, due in part at least to spontaneous gene mutations (11). A spontaneous male in an asexual population of the male strain will secrete enough hormone to initiate the production of male spheroids in the next generation. In order to grow large populations of the male strain for production of the hormone, it was necessary to grow the inoculum first in small volumes (1 parental spheroid/50 ml of Volvox medium/250 ml prescription bottle) which after 7 days' growth could be examined and those cultures discarded which showed premature male production. Usually two bottles (each containing approximately 45,000 young asexual spheroids) were added to each 2000-ml flask containing 1000 ml of Volvox medium with 0.05% sodium acetate. Thus each flask would have 90,000–100,000 small asexual spheroids whose gonidia would form male spheroids in the next generation. To insure that these spheroids would be males, 1 ml of a sterile solution containing the hormone was added to each large flask. Males were formed within 2–3 days, and sperm packets could be observed in these males 24 hr later. The flasks were kept on the lighted shelves with constant aeration until 48 hr after the sperm had been released, by which time the sperm had disintegrated. The contents of the flasks were put into plastic bags and frozen at −20°. Fluids formed under optimum conditions could be expected to stimulate 100% formation of sexual embryos in the bioassay at dilutions of 10^7 or less, becoming limiting at the 10^8 dilution with 50% or less of the embryos being sexual. However, such conditions were not always achieved, inasmuch as lack of synchrony in the inoculum would result in inhibition of later developing males by the dissolution of early males which would make the medium less conducive to the best growth.

The bioassay

The bioassay of the hormone in fluids from sexual populations of the male strain uses the HK10 female strain as the detector. Serial 1/10 dilutions are made in Volvox medium and then inoculated with 75+ young asexual spheroids of the female strain. Within 48 hr the asexual reproductive cells of the inoculum will have formed 1000+ embryos. In those dilutions where the hormone is not in limiting concentration, all, or nearly all, of the offspring will be female rather than asexual. In only one tube of a dilution series will be found a mixture of female and sexual offspring, an indication that in that dilution the hormone was in limiting concentration. All dilutions greater than this will contain no females among the offspring. Thus, one need score only the offspring in a single tube of any dilution series. Details of the assay method have been published earlier (5).

Concentration and purification procedures

The frozen fluid from male cultures was thawed in 2-liter batches, filtered through glass wool to remove large debris, and centrifuged at 10,000 × *g*. After adjusting it to pH 5, the fluid was passed rapidly (500–600 ml/hr) through a column of carboxymethyl cellulose (2.6 × 20 cm; Bio-Rad Laboratories) which had been equilibrated with 0.001 M citrate–phosphate buffer (pH 5). The very dilute nature of the medium in which the male *Volvox* had been grown made it unnecessary to dialyze the fluid prior to its passage through the column. All (99%) of the activity remained on the column.

After washing with 0.001 M citrate phosphate buffer, 0.1 M NaCl in 0.05 M citrate–phosphate buffer (pH 5) was applied to the column and the active substance was eluted in 200 ml (after discarding a 35-ml void volume). The pH of this eluant was adjusted to pH 6.5 with 1 N NaOH and the resultant salt buffer was frozen for storage.

Salt buffer from three 2-liter runs was thawed, combined, and then flash-evaporated at 40° to a volume of approximately 45 ml. This concentration resulted in a heavy white precipitate, but the activity remained in the supernatant. The concentrate was centrifuged for 10 min in a clinical centrifuge at 1500 × *g*. The supernatant was then put on a column (Pharmacia 26/100) of Sephadex G-75 with 0.1 M ammonium acetate (pH 7) as the eluant. This preparative column was run at 50 ml/hr, and the eluant collected in 10-ml fractions monitored with an ultraviolet absorptiometer (280 nm). Peaks of absorption were seen in fractions 15–20 (molecular weight >70,000), 21–30, and 35–46 (low-molecular-weight substances), but the bulk of the activity was only in fractions 21–30, reaching a maximum usually in 25 and 26. The peak of activity was slightly before the peak seen when α-chymotrypsin (molecular weight 25,000) was run on the same column as a marker protein. Fractions 21–30 of several runs were then combined, flash-evaporated to 1/10 volume and rechromatographed on the Sephadex G-75 column. The active fractions were then combined and lyophilized.

Further purification was achieved by chromatography on a Sephadex G-50 column (0.8 × 80 cm) with 0.05 M NH_4HCO_3 (pH 8.5) as equilibration buffer and eluant (2 ml/hr). The elution profile, as recorded by ultraviolet absorption (280 nm), showed one small and one large band of high-molecular-weight and one of low-molecular-weight. The active principle was in the second band of high-molecular-weight. The ultraviolet absorption coincides exactly with the activity. The active fractions were combined and lyophilized.

Characterization

Qualitatively, the white fluffy material gives strong protein reactions, sugar reaction, and a faint, but definite, phosphate reaction. On a weight basis, it contains 62% protein (standard: bovine-serum albumin) and 40.5% sugar (standard: mannose). The spectrum of the dialyzed factor at pH 8 (0.1 M Tris·HCl) shows a minimum at 252 nm ($A^{1\%}_{1cm} = 2.4$), a maximum at 273 nm ($A^{1\%}_{1cm} = 3.3$), and a shoulder at 283 nm. The spectrum has a somewhat asymmetric shape and, for a simple protein, there is an unusually high absorption in the 255- to 265-nm range ($A_{250/280} = 1.2$). At pH 13 (0.1 M NaOH) the spectral maxi-

TABLE 1. *Amino acid analysis of Volvox carteri hormone (from 55 μg of substance)*

Amino-acid residue	nmol	Residues (His = 2)
Asp:	27.6	13
Thr:	25.5	12
Ser:	34.9	16
Glu:	23.5	11
Pro:	19.5	9
Gly:	30.0	13
Ala:	28.6	13
Cys:	6.2	3
Val:	20.1	9
Met:	4.8	2
Ile:	15.8	7
Leu:	22.7	10
Tyr:	9.2	4
Phe:	7.8	4
Lys:	11.8	6
His:	4.6	2
Arg:	18.9	8–9
Trp:		1 (?)
	sum:	143–144

mum shifts to 283 nm ($A^{1\%}_{1\,cm}$ = 2.7). However, it was not possible to calculate the tryptophan content from the spectral changes (12).

The highly purified factor gives a reaction of 100% in the bioassay at a concentration of 10^{-10} g/liter and 14.4% reaction at 10^{-11} g/liter. On heating a 2 mg/ml solution of the active material in 0.05 M NH_4HCO_3 for 30 min at 60°, it retains full activity. The spectrum of the heated solution has a maximum at 260 nm ($A^{1\%}_{1\,cm}$ = 2.6) and a shoulder at 275 nm ($A^{1\%}_{1\,cm}$ = 2.2). The sex hormone is heat stable up to 80° (15 min); at 90° (15 min) 90% of the activity was lost. The biological activity is also retained after treating the material with 6 M guanidine·HCl at room temperature for 24 hr. The chaotrope even seems to stabilize the substance against heat inactivation. A low-molecular-weight cofactor could not be dissociated or split off the molecule. The active principle is not dialysable. It is salted out by 80% ammonium sulfate saturation but not adsorbed on charcoal, DEAE cellulose, or QAE Sephadex. 10% acetic acid does not precipitate the material, but it is coagulated by 10% trichloroacetic acid. Since a glycoprotein was indicated by survey analyses, several chemical data were collected and the molecular behavior of the conjugated protein was studied.

The active substance chromatographs as a single band (R_F 0.61) on cellulose thin layers (prewashed with butanol–acetic acid–water, 4:1:5) in 0.05 M NH_4HCO_3 as solvent. The activity coincides with the protein localization. The molecular weight, by comparison with standard proteins on Sephadex G-25 (13), is 25,500. On electrophoresis on cellulose thin layers at pH 6.5 it migrates as a single zone slightly towards the anode. Disc electrophoresis (staining in and destaining with 10% trichloroacetic acid) shows a diffuse weakly staining zone close to the origin and another unsharp band corresponding to a molecular weight of about 25,000 (14). The activity is spread as a zone between the two. Sodium dodecyl sulfate gel electrophoresis (15) in a 7.5% gel gave a well-defined single band at 28,400 ± 700 daltons (standards: ribonuclease, trypsin, triosephosphate dehydrogenase, bovine-serum albumin). Thus, the protein is a single chain, not composed of subunits. The relatively high value in comparison with the other data on molecular weight is probably due to the high carbohydrate content of the glycoprotein (16). This also is indicated by a relative shift of the mobility in a 10% gel. Sucrose gradient centrifugation (17) gives a molecular weight of 26,000, assuming a partial specific volume of 0.700. On sedimentation velocity analysis, the highly purified preparations (concentration = 0.65 to 2.7 mg/ml in 0.05 M NH_4HCO_3) form a well-defined symmetrical slow band ($s^*_{20,w}$ = 1.57 ± 0.07) and another fairly sharp and symmetrical faster moving band ($s^*_{20,w}$ = 2.7). Both of them contain the activity. The diffusion coefficient for the slow band was determined in 0.05 M NH_4HCO_3 as $D^*_{20,solv.}$ = 4.16 ± 0.02 × 10^{-7}. From these hydrodynamic data a molecular weight of 30,600 ± 5% is calculated and a disymmetry constant of f/f_0 = 1.66 is derived, representing a rather elongated, asymmetric molecule. It readily forms dimers and also apparently polymers at higher concentrations. It seems that the active proteide is in concentration-dependent, reversible equilibrium with multiple aggregates, whereas on column chromatography, when the concentration was about 0.2 mg/ml, only the monomer is eluted.

The amino-acid analysis shows no unusual amino acids (Table 1). Based on histidine as unit, the minimal molecular weight is 7750. There are 6 lysine and 8–9 arginine residues; tryptic digestion after performic acid oxidation (18) shows a minimum of 12 and a maximum of 15 tryptic peptides. There are three strongly anionic peptides, derived from cysteine stretches. Thus, the molecular weight of the protein moiety is around 15,000 (2 × 7750), corresponding to an overall molecular weight of 28,200 under the assumption of a 55:45 protein to sugar ratio. The amount of amino sugars, as revealed in the amino-acid analyzer, corresponds to about 5% of the total sugars. No N-terminal amino acid could be detected; the amino terminal is either closed by a small substituent or by the glycosidic moiety. The peptide was iodinated by ICl (iodine monochloride) in glycine buffer (pH 9.5) (19), almost without loss of biological activity. Gas chromatographic sugar analysis after methanolysis, acetylation, and trimethylsilylation (20) of the material gave (internal standard: pertrimethylsilylmannitol) arabinose (6.6%), xylose (25.5%), mannose (15.6%), galactose (4.6%), glucose (32.5%), unknown peak (*N*-acetylgalactosamine?) (11.3%), and *N*-acetylglucosamine (3.9%).

These data taken together demonstrate that the sexual hormone produced by the male population of *Volvox carteri* f. *nagariensis* is a glycoprotein of 27,500–30,000 daltons, composed of only one polypeptide chain which has a typical composition, except for a very low value for tryptophan and a strikingly high carbohydrate content. The main components of the nonprotein material are xylose and glucose. The ratio: pentoses to hexoses to aminohexoses is 2:3.5:1. The hormone tends to aggregate reversibly at higher concentrations in 0.05 M NH_4HCO_3.

Discussion

Numerous instances of apparent chemical control of the sexual processes in plants have been reported, but few have been analyzed in any detail (21). In some plants the substances serve to bring together gametes of the compatible mating types or sexes, e.g., *Allomyces* (22), *Ectocarpus* (23), and *Chlamydomonas* (24). In others, the differentiation of the sexual struc-

tures which produce the gametes depends on the secretion of active compounds by one or both compatible individuals. Thus, in *Mucor* (25) and *Achlya* (26) the hormones initiate the differentiation of special sex organs on siphonaceous plant bodies, while in certain ferns (27) the hormones effect a special pattern of cell cleavage and differentiation resulting in the formation of antheridia, the multicellular male reproductive organs. The hormone secreted by the male *Volvox* is similar in effect to that of ferns, but the hormones are unrelated chemically. In the ferns the antheridiogens are thought to be related to the gibberellins, and, indeed, in some species the response to both antheridiogens and various gibberellins is the same. The antheridiogens from different ferns do not show the extreme species-specificity seen in the sexual hormones from the various species of *Volvox* (27, 28).

The morphogenetic hormone of *Volvox carteri f. nagariensis* is a medium sized glycoprotein of high (45%) carbohydrate content whose overall composition bears similarities to certain other plant glycoproteins (29) in its lack of neuraminic and uronic acids. No fucose and other deoxy sugars were detected; however, an unknown sugar (tentatively identified as the —gas chromatographically similar—*N*-acetylgalactosamine), was found present in appreciable quantities. The amount of amino sugars, as determined with the amino-acid analyzer, accounts only for the amount of *N*-acetylglucosamine estimated independently by gas chromatography. If the unknown sugar were *N*-acetylgalactosamine, the relative amounts of the two amino hexoses would be inversely proportional to those of the corresponding hexoses. The amino-acid analysis showed a relatively low content in sulfur amino acids. Whereas the protein moiety consists of but one polypeptide chain, it is not clear whether the carbohydrate moiety is also a single polymer. Although the binding amino acids are not known, the high content of acidic amino acids and of serine is conspicuous. There is no terminal amino group. It might be one site of carbohydrate binding or blocked by acylation. The absorbance of the hormone at 280 nm corresponds well with the amount of aromatic amino acids found, assuming at the most only one tryptophan, which, however, has so far not been detected independently. The absorbance of the material in the 250- to 270-nm range is higher than would be expected for a simple glycoprotein. Changes of the spectrum with pH may be explained by the presence of a very tightly bound heterocyclic (nucleotidic) base. However, it was not possible to liberate and identify such a compound. Other striking features are the relatively low sedimentation constant and the similarly low diffusion constant, which compensate to a molecular weight comparable to that found by independent means. The frictional ratio (f/f_0) points to a rather elongate shape such as that of other glycoproteins of biological activity. The sites of dimerization and further aggregation are probably charged parts of the molecule.

Nothing is known about the biochemistry of the response initiated by the sexual hormone in *Volvox*. It would appear to function only as an initiator of the pathway of development leading to a sexual embryo, for it is equally effective in both the male and the female strains; and it has been clearly demonstrated that the particular events of development in the embryogeny are under the control of various genetic loci. The identification of two genetic loci, one linked to sex, which result in the formation of a sexual embryo without the addition of the sexual hormone, has been used to postulate a scheme of biochemical action in which the hormone serves as a co-repressor (11). With the present demonstration of the chemical nature of the hormone and the possibility of iodination without loss of biological activity, it may now be possible to test this hypothesis using labelled hormone, immunological procedures, etc.

In the bioassay of the highly purified hormone a 14.4% reaction was obtained at a concentration of 10^{-11} g/liter or 3×10^{-16} M, assuming a molecular weight of 30,000. Approximately 100 gonidia per ml are involved in the bioassay, and thus it figures that 1800 molecules per gonidium resulted in the 14.4% reaction. The actual number of molecules necessary to effect the reaction in a gonidium is probably much lower. Earlier experiments have shown that increasing the inoculum per ml 20-fold did not change the level of the reaction; larger numbers of gonidia have not been tried inasmuch as such numbers result in problems of limiting CO_2, illumination, and media composition. Pall (30) has published data from studies of the fraction of sexual induction as a function of inducer concentration and he concluded that only 2 molecules of the hormone are actually required to make the gonidium form a sexual embryo rather than an asexual one.

Volvox offers possibilities for investigations of the control of development and differentiation in a multicellular organism using biochemical approaches generally more applicable to populations of microorganisms. Furthermore, the ease with which *Volvox* can be cultivated and its sexual phases evoked allows one to investigate genetic phenomena in this haploid autotrophic organism. The development of the many approaches to problems of cellular differentiation will, of course, depend on investigators with different interests and competencies; therefore, the male and female strains of *Volvox carteri f. nagariensis* have been deposited in the Culture Collection of Algae at Indiana University, Bloomington, for unrestricted distribution (31).

R. C. Starr wishes to thank Prof. Dr. J. Straub, Director of the Max-Planck-Institut für Züchtungsforschung, Cologne, for providing facilities and supplies necessary to carry on much of this research. Continuing assistance from Drs. D. Müller and O. Schieder during his stay at the Institut is gratefully acknowledged. We thank Mr. E. Bause and Dr. U. Menge for help with the sugar, amino acid, and ultracentrifuge analyses. Financial support to R. C. Starr for the year's stay at the Institut was from the sabbatical leave program of Indiana University and through an award under the "Sonderprogramm zur Förderung der fachbezogenen Zusammenarbeit zwischen Forschungsinstituten in der Bundesrepublik Deutschland und in den Vereinigten Staaten von Amerika" administered by the Alexander von Humboldt-Foundation for the Federal Republic of Germany.

1. Dobell, C. (1932) *Antony van Leeuwenhoek and His Little Animals* (Staples Press, London), pp. 256–262.
2. Powers, J. H. (1907) "New forms of *Volvox*," *Trans. Amer. Microsc. Soc.* **27**, 123–149; Powers, J. H. (1908) "Further studies in *Volvox*, with descriptions of three new species," *Trans. Amer. Microsc. Soc.* **28**, 141–175.
3. Darden, W. H., Jr. (1966) "Sexual differentiation in *Volvox aureus*," *J. Protozool.* **13**, 239–255.
4. Starr, R. C. (1968) "Cellular differentiation in *Volvox*," *Proc. Nat. Acad. Sci. USA* **59**, 1082–1088.
5. Karn, R. W. (1972) Differentiation of reproductive cells in *Volvox obversus*. Ph.D. thesis, Indiana University, Bloomington; Kochert, G. (1968) "Differentiation of reproductive cells in *Volvox carteri*," *J. Protozool.* **15**, 438–452; McCracken, M. D. & Starr, R. C. (1970) "Induction and development of reproductive cells in the K-32 strains of *Volvox rousseletii*," *Arch. Protistenk.* **112**, 262–282; Starr, R. C. (1969) "Structure, reproduction, and differentiation in *Volvox carteri f.*

nagariensis Iyengar, strains HK 9 & 10," *Arch. Protistenk.* 111, 204–222; Starr, R. C. (1972) "Sexual reproduction in *Volvox dissipatrix*," *Abstr. Br. Phycol. J.* 7, 284; Vande Berg, W. J. & Starr, R. C. (1971) "Structure, reproduction, and differentiation in *Volvox pigus* and *Volvox powersii*," *Arch. Protistenk.* 113, 195–219.

6. Starr, R. C. (1970) "Control of differentiation in *Volvox*," in *Changing Syntheses in Development*, ed. Runner, M. N., *Develop. Biol. Suppl.* 4, 59–100.
7. Starr, R. C. (1972) "Sexual reproduction in *Volvox dissipatrix*," *Abstr. Br. Phycol. J.* 7, 284.
8. Darden, W. H., Jr. (1970) "Hormonal control of sexuality in the genus *Volvox*," in *Phylogenesis and Morphogenesis in the Algae*, ed. Frederick J. F. and Klein, R. M., *Ann. N.Y. Acad. Sci.* 175, 757–763.
9. Ely, T. H. & Darden. W. H. (1972) "Concentration and purification of the male-inducing substance from *Volvox aureus* MS," *Microbios* 5, 51–56.
10. Provasoli, L. & Pintner, I. J. (1959) "Artificial media for freshwater algae; problems and suggestions," in *The Ecology of Algae*, eds. Tryon, C. A. & Hartman, R. T. (Spec. Publ. No. 2, Pymatuning Lab. of Field Biology, University of Pittsburgh), pp. 84–96.
11. Starr, R. C. (1972) "A working model for the control of differentiation during development of the embryo of *Volvox carteri* f. *nagariensis*," *Soc. Bot. Fr., Memoires* 1972, pp. 175–182.
12. Bencze, W. L. & Schmid, K. (1957) "Determination of tyrosine and tryptophane in protein," *Anal. Chem.* 25, 1193–1196.
13. Andrews, P. (1965) "The gel-filtration behavior of proteins relative to their molecular weight over a wide range," *Biochem. J.* 96, 595–606.
14. Hedrick, J. C. & Smith, A. J. (1968) "Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis," *Arch. Biochem. Biophys.* 126, 155–164.
15. Weber, K. & Osborn, M. (1969) "The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis," *J. Biol. Chem.* 244, 4406–4412.
16. Bretscher, M. A. (1971) "Major human erythrocyte glycoprotein spans the cell membrane," *Nature New Biol.* 231, 229–230.
17. Martin, R. G. & Ames, B. N. (1961) "A method for determining the sedimentation behavior of enzymes: Application to protein mixtures," *J. Biol. Chem.* 236, 1372–1379.
18. Rao, D. R., Beyreuther, K. & Jaenicke, L. (1973) "A comparative study of pig and sheep brain glutamine synthetase: Tryptic peptides and thiol groups," *Eur. J. Biochem.* 35, 582–592.
19. Roholt, O. A. & Pressman, D. (1972) "Iodination—Isolation of peptides from the active site," in *Methods in Enzymology*, eds. Hirs, C. W. H. & Timasheff, S. N. (Academic Press, New York), Vol. 25, Part B, pp. 438–449.
20. Donike, M. (1969) "*N*-Methyl-*N*-trimethylsilyl-trifluoracetamid, ein neues Silylierungsmittel aus der Reihe der silylierten Amide," *J. Chromatogr.* 42, 103–104.
21. Machlis, L. (1972) "The coming of age of sex hormones in plants," *Mycologia* 64, 235–247.
22. Machlis, L., Nutting, W. H., Williams, M. W. & Rapoport, H. (1966) "Production, isolation, and characterization of sirenin," *Biochemistry* 5, 2147–2152.
23. Müller, D. G., Jaenicke, L., Donike, M. & Akintobi, T. (1971) "Sex attractant in a brown alga; chemical structure," *Science* 171, 815–817.
24. Wiese, L. (1969) "Algae," in *Fertilization*, eds. Metz, C. B. & Monroy A. (Academic Press, New York), Vol. 2, pp. 135–188.
25. van den Ende, H. & Stegwee, D. (1971) "Physiology of sex in Mucorales," *Bot. Rev.* 37, 22–36.
26. Barksdale, A. W. (1969) "Sexual hormones of *Achlya* and other fungi," *Science* 166, 831–837.
27. Voeller, B. R. & Weinberg, E. S. (1969) "Evolutionary and physiological aspects of antheridium induction in ferns," in *Current Topics in Plant Science*, ed. Gunckel, J. E. (Academic Press, New York), pp. 79–93.
28. Endo, M., Nakanishi, K., Naf, U., McKeon, W. & Walker, R. (1972) "Isolation of the antheridiogen of *Anemia phyllitides*," *Physiol. Plant.* 26, 183–185.
29. Gottschalk, A. & Buddecke, E. (1972) "Occurrence and distribution of glycoproteins," in *Glycoproteins*, ed. Gottschalk, A. (Elsevier Publishing Co., Amsterdam), 2nd ed., pp. 529–564.
30. Pall, M. (1973) "Sexual induction in *Volvox carteri*: a quantitative study," *J. Cell Biol.* 59, 238–241.
31. Starr, R. C. (1971) "The culture collection of algae at Indiana University—additions to the collection July 1966–July 1971," *J. Phycol.* 7, 350–362.

Trophic Conversion of an Obligate Photoautotrophic Organism Through Metabolic Engineering

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Most microalgae are obligate photoautotrophs and their growth is strictly dependent on the generation of photosynthetically derived energy. We show that the microalga *Phaeodactylum tricornutum* can be genetically engineered to thrive on exogenous glucose in the absence of light through the introduction of a gene encoding a glucose transporter (*glut1* or *hup1*). This demonstrates that a fundamental change in the metabolism of an organism can be accomplished through the introduction of a single gene. This also represents progress toward the use of fermentation technology for large-scale commercial exploitation of algae by reducing limitations associated with light-dependent growth.

Photosynthetic algae are dominant producers in aquatic environments, accounting for a substantial proportion of worldwide O_2 production and CO_2 fixation (*1*, *2*). They are also a component of feed for aquaculture and produce numerous valuable compounds including pigments (e.g., β-carotene, phycobiliproteins), oils (e.g., docosahexaenoic acid), and stable isotope-labeled biochemicals (e.g., [^{13}C]glucose); they also have potential in the discovery of new pharmaceuticals (*3*).

Commercial-scale cultivation of photosynthetic microalgae is typically performed in large, open outdoor ponds. This mode of growth, although it exploits natural sunlight for the production of energy (*3*, *4*), is associated with numerous disadvantages. Contaminants invade pond cultures, and seasonal and diumal variations in temperature and light conditions make it difficult to predict both growth rates and final culture densities. Self-shading restricts light availability, severely limiting biomass production, and low cell densities prevent efficient harvesting of the cells. Together, these factors have restricted large-scale cultivation of microalgae to a small subset of genera that includes *Spirulina* and *Dunaliella*.

A strategy that can improve the efficiency and reduce the cost of microalgal biomass production involves heterotrophic growth of algae in conventional microbial fermenters (in the absence of light) (*3*–*5*). Fermenters provide controlled, sterile growth conditions that maximize productivity. Glucose or other forms of organic carbon, rather than light, supply energy and reducing equivalents. However, the use of fermentation technology is limited, because most microalgae are obligate photoautrophs and are unable to grow on fixed carbon compounds (*6*).

These considerations suggest advantages to genetically engineering microalgal metabolism for high rates of heterotrophic growth. One microalga that can be genetically modified by transformation (*7*–*9*), but is unable to grow heterotrophically (*6*, *10*, *11*), is the diatom *Phaeodactylum tricornutum* (UTCC646). We attempted a trophic conversion of this alga by transforming it with genes encoding glucose transporters. The transporter genes used included *Glut1* from human erythrocytes (*12*); *Hup1* from the microalga *Chlorella kessleri* (*13*); and *Hxt1*, *Hxt2*, and *Hxt4* from *Saccharomyces cerevisiae* (*14*). The coding regions of these genes were inserted into the *P. tricornutum* transformation vector pPha-T1 (*8*). A construct (Glut1-GFP) was also generated in which the green fluorescent protein gene, *GFP*, was fused to the 3′ end of the *Glut1* gene. Plasmids were introduced into *P. tricornutum* by using biolistic procedures, and transformants were selected for zeocin resistance in the light (*8*). The transformants were then transferred to solid or liquid medium containing 0.1 or 1.0% glucose, placed in complete darkness, and monitored for growth (*15*).

The *P. tricornutum* cell lines transformed with the *Glut1* gene (19 of 28) exhibited rates of glucose transport (*16*) between 0.2 and 13 nmol glucose/min for 10^8 cells (19 of 28 primary transformants). Cell lines with uptake rates of ≥1.6 nmol glucose/min for 10^8 cells (11 of 28) grew on glucose in the dark. For *Hup1*-containing transformants, 14 of 25 exhibited glucose uptake rates between 0.06 and 1.9 nmol glucose/min for 10^8 cells. Cell lines with uptake rates of ≥0.29 nmol/min for 10^8 cells (11 of 25) grew in the dark. None of the cell lines transformed with the control vector or the yeast transporter genes exhibited detectable glucose uptake (*17*). The inability of the transformants to express functional Hxt protein may reflect differences in codon usage between yeast and *P. tricornutum* (*8*).

A detailed characterization was performed on a number of the *Glut1* transformants, including *Glut1-17* and *Glut1GFP-40*. Monospecific antibodies against the Glut1 polypeptide and GFP were used to demonstrate accumulation of Glut1 or the Glut1-GFP fusion protein in transformed cell lines (*18*). Membranes of the *Glut1-17* transformant contained two prominent polypeptides that reacted with Glut1-specific antibodies (Fig. 1); no cross-reacting material was present in the soluble phase of the cell (*17*). These polypeptides had molecular masses of 44 and 39 kD, which is less than the native protein (~55 kD) synthesized in human erythrocytes (Fig. 1, compare lanes B and G1-17), but which is close to the size of unglycosylated Glut1 (38 kD) (*19*). This implies that Glut1 synthesized in *P. tricornutum* is glycosylated differently from that in human erythrocytes. The Glut1-GFP fusion protein present in the *Glut1GFP-40* transformant had a molecular mass of ~75 kD, which is slightly smaller than the expected mass of Glut1-GFP (82 kD), presumably resulting from glycosylation differences.

Both the *Glut1-17* and *Glut1GFP-40* transformants showed high rates of glucose uptake (*16*) (Fig. 2). The *Glut1-17* transformant had a K_m for glucose of 1.2 mM and a V_{max} of 7.6 nmol glucose/min for 10^8 cells; *Glut1GFP-40* had a K_m of 1.0 mM and a V_{max} of 13 nmol glucose/min for 10^8 cells. The K_m values for glucose in the transformants are similar to those (1 to 2 mM) measured for human erythrocytes (*12*). Differences in the V_{max} between transformants probably reflect different levels of expression of the *Glut1* gene, which could depend on the site of integration into the diatom genome. In the presence of 5×10^{-6} units of cytochalasin B per milliliter, a specific inhibitor of Glut1-dependent transport (*20*), glucose uptake was reduced to undetectable levels (*17*). These results demonstrate that Glut1 facilitates glucose transport into *P. tricornutum* cells and that the affinity of the transporter for glucose is essentially the same as in human erythrocytes.

To determine the subcellular location of the Glut1 protein in transformed lines, the *Glut1GFP-40* strain was examined for GFP fluorescence by confocal microscopy (*21*). Untransformed cells showed strong chlorophyll fluorescence, but low fluorescence in the green channel (Fig. 3, A, B, and C).

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Transformed cells containing the *GFP* gene exhibited GFP fluorescence consistent with localization in the cytosol and the lumen of the cell nucleus (Fig. 3D). A similar distribution of soluble GFP in plant cells has been observed (*22*, *23*). In contrast, *Glut1GFP-40* showed fluorescence associated with the extreme periphery of the cells (Fig. 3E). These results demonstrate that the Glut1 protein targets GFP to the cytoplasmic membrane, a pattern consistent with exclusive localization of the chimeric protein to the membrane fraction and the function of Glut1 in membrane-associated transport.

Commercial exploitation of metabolically engineered diatoms requires high rates of glucose-dependent growth and the achievement of high cell densities. Growth of the *Glut1-17* transformant was measured in the light and dark in liquid medium supplemented with glucose (*15*). As shown in Fig. 4, both untransformed cells and the *Glut1-17* transformant grown in the light without glucose reached the same cell densities (3×10^7 cells/ml). The addition of glucose did not change the growth characteristics of the untransformed strain. In contrast, the transformed strain attained a cell density about five times as high. Untransformed cells are unable to grow in the dark in the presence of glucose, but *Glut1-17* grew at the same rate and to the same cell density in the presence of glucose in the light or dark. As the cultures became more dense, and as light absorption was attenuated by self-shading, the rate of growth of *Glut-17* in the presence of glucose exceeded that of untransformed cells. If heterotrophic growth is conducted in a microbial fermenter with continuous addition of glucose and other nutrients, the density attained by Glut-17 exceeded that of untransformed cells 15 times (*17*), reaching densities of 5×10^8 cells/ml.

Most diatoms do not have the capacity to grow in the absence of light on exogenous glucose (*6*). This report demonstrates that trophic conversion of the obligate photoautotrophic diatom, *P. tricornutum*, can be achieved by transforming the alga with a single gene encoding a glucose transporter. Functionality of the algal and human glucose transporters in *P. tricornutum* suggests that the heterologous proteins are correctly targeted and inserted into diatom membranes. The inability of the diatom to glycosylate Glut1 normally suggests that the carbohydrate moiety may not play a critical role in targeting Glut1 to the cytoplasmic membrane or in transport function. *Hup1* has also been expressed in the microalgae *Volvox* (*24*) and *Cylindrotheca* (*25*), but neither were able to grow heterotrophically.

Trophic conversion by introduction of a glucose transporter requires that the complete glycolytic pathway is present within the cells and that it can support a high flux of metabolites. Photosynthetic organisms transfer fixed carbon out of plastids into the cytoplasm where it can be metabolized into hexose sugars; these sugars can then be used to support growth or stored as polysaccharides. Diatoms synthesize chrysolaminarin, a β-1,3-glucan polymer, as the primary carbohydrate storage compound (*26*). The catabolism of this glucose polymer would require glycolysis. Hexokinase activity, required for conversion of glucose to glucose-6-phosphate, the first metabolite of the glycolytic pathway, has also been reported in diatoms (*11*). Thus all of the necessary activities for glucose metabolism already exist in diatom cells. As a result, exogenous glucose entering the cell can be metabolized at a high flux, allowing the cells to thrive in the absence of light.

The trophic conversion of microalgae such as diatoms is a critical first step in engineering algae for successful large-scale cultivation using microbial fermentation technology. In addition to providing a means for maintaining culture conditions, glucose and other nutrients can be continuously provided to maximize productivity. The use of fermentation technology elim-

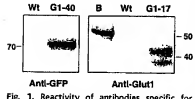

Fig. 1. Reactivity of antibodies specific for Glut1 or GFP to membrane proteins extracted from untransformed and transformed cell lines. The proteins were resolved by SDS–polyacrylamide gel electrophoresis after solubilization of total membranes from wild-type (Wt), untransformed cells; G1-40, the *Glut1GFP-40* transformant; B, human erythrocytes; G1-17, the *Glut1-17* transformant. The antibodies used were specific for GFP (left panel) or Glut1 (right panel).

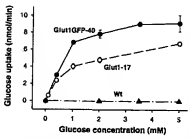

Fig. 2. Uptake of glucose by 10^8 cells of transformants *Glut1GFP-40* and *Glut1-17*, compared with wild-type untransformed cells (Wt). Glucose uptake was assayed as described (*16*).

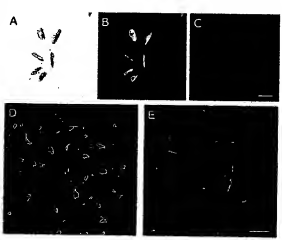

Fig. 3. Localization of Glut1 in *P. tricornutum* transformed with the chimeric gene encoding the Glut1-GFP fusion protein. The top panel shows a transmitted light image of untransformed *P. tricornutum* cells (**A**), fluorescence from the cells in the red channel (**B**), representing chlorophyll; and fluorescence in the green channel (**C**). The lower panel shows fluorescence in the green channel of cells that were transformed with the *GFP* gene (**D**), and of cells transformed with the chimeric *Glut1-GFP* gene [(**E**), strain designated Glut1-40]. Scale bars, 10 μm.

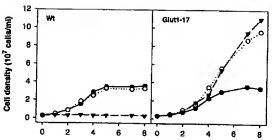

Fig. 4. Growth of untransformed cells and *Glut1-17* under different conditions. The left panel shows the growth of untransformed, wild-type cells (Wt), and the right panel shows the growth of *Glut1-17*. Growth was in the light (filled circles), in the light plus glucose (open circles), or in the dark plus glucose (filled triangles).

inates contamination by microbes, which is an important criterion for maintaining food industry standards. Fermentative growth of naturally heterotrophic microalgae has resulted in dry biomass accumulation to 100 g/liter (*3*, *27*), which is 10 to 50 times the yields obtained by using light-dependent culture systems. Fermentation-based systems can reduce production costs of microalgae by an order of magnitude relative to that incurred by photosynthesis-based production; cost reduction analyses factor in expenses for both fixed-carbon supplementation and equipment operation (*28*). Commercial benefits of fermentation-based systems result from increased biomass, productivity, harvesting efficiency, and reduced losses from contamination. The ability to grow microalgae heterotrophically increases the feasibility for developing a large range of new algal products.

Marine ecosystems also depend on diatoms, which contribute substantially to the reduction of inorganic carbon in marine habitats. Such a contribution may increase substantially as the ecology of oceanic environments is altered (*29*–*32*). The exploitation of diatoms that can be genetically manipulated and that can grow heterotrophically will facilitate the use of mutants to augment our understanding of both photosynthesis and other metabolic pathways that are essential for competing in marine ecosystems.

References and Notes

1. P. Tréguer *et al.*, *Science* **268**, 375 (1995).
2. S. W. Chisholm, *Nature* **407**, 685 (2000).
3. K. E. Apt, P. Behrens, *J. Phycol.* **35**, 215 (1999).
4. M. A. Borowitzka, *J. Biotech.* **70**, 313 (1999).
5. F. Chen, *Trends Biotechnol.* **14**, 421 (1996).
6. M. R. Droop, in *Algal Physiology and Biochemistry*, W. D. P. Stewart, Ed. (Univ. of California Press, Berkeley, 1974), pp. 530–559.
7. K. E. Apt, P. G. Kroth-Pancic, A. R. Grossman, *Mol. Gen. Genet.* **252**, 572 (1996).
8. L. A. Zaslavskaia, J. C. Lippmeier, P. G. Kroth, A. R. Grossman, K. E. Apt, *J. Phycol.* **36**, 379 (2000).
9. A. Falciatore, R. Casotti, C. Leblanc, C. Abrescia, C. Bowler, *Mar. Biotechnol.* **1**, 239 (1999).
10. K. E. Cooksey, *J. Phycol.* **10**, 253 (1974).
11. J. A. Hellebust, J. Lewin, in *The Biology of Diatoms*, D. Werner, Ed. (Univ. of California Press, Berkeley, 1977), pp. 169–197.
12. M. Mueckler, R. C. Hresko, M. Sato, *Biochem. Soc. Trans.* **25**, 951 (1997).
13. N. Sauer, W. Tanner, *FEBS Lett.* **259**, 43 (1989).
14. A. L. Kruckeberg, *Arch. Microbiol.* **166**, 283 (1996).
15. After 4 weeks in the dark, the transformants that grew were restreaked and maintained on 1.0% glucose. Liquid cultures were grown with 1.0% glucose at 20°C on an orbital shaker. All characterized transformants were generated from independent particle bombardments. Cells were grown at 20°C with continuous illumination at 75 μmol photons m^{-2} s^{-1} in Provasoli's enriched seawater medium with 10× the nitrogen and phosphorus by using instant Ocean artificial seawater, at 0.5× concentration. Glucose was maintained between 5 and 10 g/liter. Growth rates were determined in 250-ml flasks (50 ml of media) with silicon foam closures. Daily samples measured cell numbers and nutrients. Flasks were stirred at 100 rpm. Fermentations were done in a 2-liter Applikont vessel by using an agitation rate of 100 rpm, dissolved oxygen was maintained at >20% saturation.
16. Cells in logarithmic phase growth were harvested, washed two times, and resuspended in fresh medium. Assays were initiated by adding unlabeled glucose and [D-^{14}C]glucose to 0.05 μCi/ml; the cells were maintained in the light. Samples were removed at 0, 2, 5, 10, and 15 min after the addition of labeled glucose. The cells were collected by filtration, washed with medium containing 1.0% unlabeled glucose, and transferred to scintillation vials.
17. L. A. Zaslavskaia *et al.*, unpublished data.
18. The cells were broken by using a BeadBeater by two cycles at full speed on ice. Cell membranes were pelleted by centrifugation at 100,000*g* for 30 min, solubilized in 2.0% SDS, resolved on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes.
19. T. Asano *et al.*, *J. Bio. Chem.* **266**, 24632 (1991).
20. L. L. Lu, A. Lundqvist, C. M. Zeng, C. Lagercquist, P. Lundahl, *J. Chromatogr.* **776**, 81 (1997).
21. Confocal microscopy was performed using a Nikon 60× N.a. = 1.2 water immersion objective on a Nikon TMD 200 inverted microscope outfitted with a BioRad MRC 1024 confocal head mounted in a Koehler configuration. ECFP was excited at 488 nm and visualized with a 522/25-nm bandpass filter. Plastid autofluorescence was excited at 456 nm and visualized with a 585-nm-long pass filter.
22. W. Chu *et al.*, *Curr. Biol.* **6**, 325 (1996).
23. J. Hasselt, K. R. Simmering, D. C. Prasher, S. Hodge, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 2122 (1997).
24. A. Hallmann, M. Sumper, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 669 (1996).
25. H. Fischer, I. Robl, M. Sumper, N. Kroger, *J. Phycol.* **35**, 113 (1999).
26. P. G. Roessler, *J. Phycol.* **23**, 494 (1987).
27. J. A. Running, R. J. Huss, P. T. Olson, *J. Appl. Phycol.* **6**, 99 (1994).
28. R. J. Radmer, B. C. Parker, *J. Appl. Phycol.* **6**, 93 (1994).
29. J. A. Raven, P. G. Falkowski, *Plant Cell Environ.* **22**, 741 (1999).
30. M. R. Landry, *et al.*, *Marine Ecol. Progr. Ser.* **201**, 57 (2000).
31. E. Boyle, *Nature* **393**, 733 (1998).
32. S. Takeda, *Nature* **393**, 777 (1998).

33. We thank D. Kyle and T. Allnutt for encouragement in the early parts of this work. Supported by NSF DMA-9560125 and funds from the Carnegie Institution of Washington.

20 February 2001; accepted 3 May 2001

Telomere Position Effect in Human Cells

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In yeast, telomere position effect (TPE) results in the reversible silencing of genes near telomeres. Here we demonstrate the presence of TPE in human cells. HeLa clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones. The dependence of TPE on telomere length provides a mechanism for the modification of gene expression throughout the replicative life-span of human cells.

Most normal human cells lack the enzyme telomerase, which maintains telomeres, and as a consequence, telomeres shorten with each division until the cells reach replicative senescence (the Hayflick limit). This growth arrest is mediated by p53 and has been suggested to be the result of a DNA damage response to telomeres that have become too short (*1*–*3*). No mechanism has been demonstrated in vertebrates that can account for differences between young and old (but not yet senescent) cells. In *Saccharomyces cerevisiae*, telomere position effect (TPE) can result in the reversible silencing of a gene near a telomere by a mechanism that depends both on telomere length and on the distance to the gene (*4*–*6*). Because telomeres in most human cells shorten with age, TPE would provide a mechanism to incrementally alter phenotype with increasing cellular age (*7*). However, previous efforts to identify TPE in mammalian cells have not been successful (*8*–*10*). We demonstrate here the presence of TPE in human cells and that the strength of the silencing effect is dependent on telomere length.

We seeded de novo telomere formation in (telomerase-positive) HeLa cells by introducing a linear plasmid containing a luciferase reporter adjacent to 1.6 kb of telomere repeats (Web fig. 1) (*11*). Integration of a repeat-containing plasmid can result in breakage of the chromosome, followed by extension of the plasmid telomeric sequences by telomerase and loss of the distal chromosome fragment (*12*). Clones with a telomeric reporter were identified by Southern blotting of purified telomeres (Fig. 1A and Web fig. 2) (*11*) and confirmed by in situ hybridization (Fig. 1B). The mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequence) was estimated from Southern blots to be between 1.5 and 2 kb. Control clones were generated by transfection of an other-

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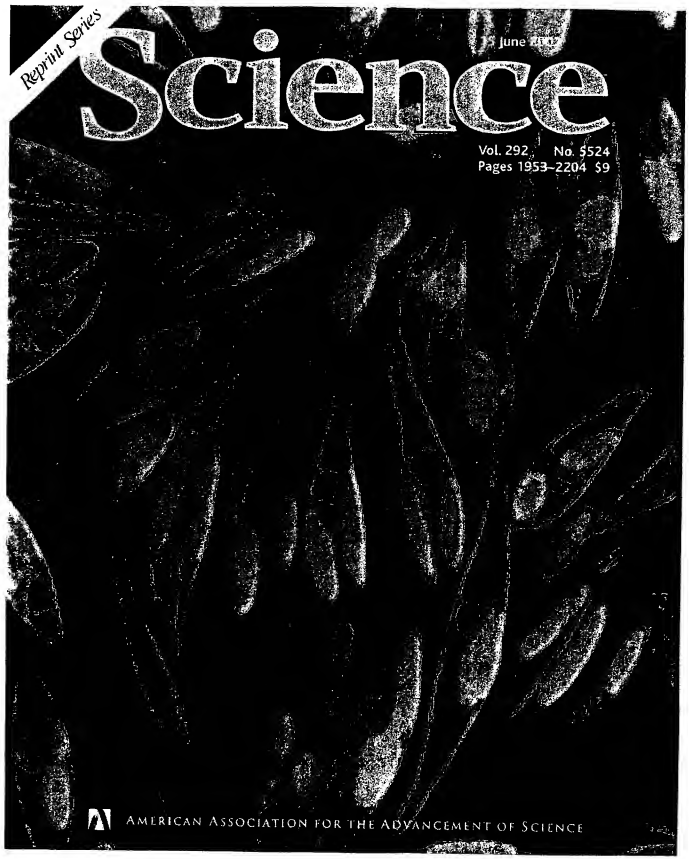
Reprint Series
Science
15 June 2001
Vol. 292 No. 5524
Pages 1953–2204 $9
AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE

J. Phycol. 37, 657–658 (2001)

藻類要聞 ✲ 繁楷體 - Algues ✲ Avancées récentes

- Algas ✲ Alto interés - Algae ✲ Highlights -

ВОДОРОСЛИ ✲ ВАЖНЕЙШИЕ МОМЕНТЫ - أخبار ✲ الطحالب

THE SWEETNESS OF DIATOM MOLECULAR ENGINEERING

Diatoms are a large group of unicellular microalgae that are ubiquitous in marine and freshwater habitats. Almost all diatom species are photoautotrophic organisms. Because seasonal phytoplankton blooms are normally dominated by diatoms, they contribute a large proportion of the primary biological production in the oceans (Smetacek 1998). Although the "normal life" of a diatom is autotrophic (i.e. independent of exogenous organic molecules), there are quite a number of diatom species known to be capable of active uptake of small organic molecules such as acids, amino acids, and monosaccharides. Some diatom species are even able to live as facultative heterotrophs in the absence of light, albeit with significantly reduced growth rates. Others, although they metabolize the organic molecules taken up, are unable to grow in the dark (Hellebust and Lewin 1977). These observations suggest that diatom metabolism is optimized for photoautotrophic growth. It, therefore, comes as a rather unexpected and very interesting result that Zaslavskaia and colleagues (2001) were able to generate by genetic engineering a facultatively heterotrophic diatom that grows in the dark. What's more, the engineered diatoms grow at normal rates and to higher cell densities than those obtained by mere photoautotrophic growth. Remarkably, creation of this "superior diatom" was achieved by introducing into *Phaeodactylum tricornutum* only a single gene (*glut-1*) that encodes the glucose transporter protein (glut-1) from human erythrocytes. How can this small change in the genome have such a dramatic effect on the diatom's physiology?

Phaeodactylum tricornutum wild-type cells cannot utilize exogenic glucose because they lack a glucose transporter system in the plasma membrane. In contrast, *glut-1* transformants that express and correctly target the glut-1 protein to the plasma membrane can take up glucose and then pass it on to glycolystic and other metabolic pathways. However, being able to take up exogenic glucose is not sufficient for heterotrophic growth of *P. tricornutum*. Zaslavskaia et al. (2001) have shown that, of the many different transformed cell lines expressing the heterologous glucose transporter, only about 60% can live heterotrophically. Transformants must exceed a minimum rate of glucose uptake to supply the cells with a sufficient amount of reduced carbon molecules to allow proliferation in the dark. Because the glucose uptake rate is probably directly correlated with the concentration of active glucose transporter molecules in the plasma membrane, this result suggests that trophic conversion of *P. tricornutum* is critically dependent on the efficiency of the expression system used in the transformation. This probably explains why an earlier attempt to convert the autotrophic diatom *Cylindrotheca fusiformis* into a facultative heterotroph failed. The maximum glucose uptake rates of *C. fusiformis* transformants (wild-type cells lack a glucose uptake system) expressing the glucose transporter H^+/hexose cotransporter (HUP-1) from the green alga *Chlorella kessleri* were significantly lower (Fischer et al. 1999) than the minimum rate required for heterotrophic growth of *P. tricornutum* transformed with HUP-1 (Zaslavskaia et al. 2001).

The successful transformation experiments by Zaslavskaia et al. (2001) in *P. tricornutum* do not come "out of the blue." Many tools (selection marker genes, reporter genes) for molecular engineering of this diatom were already available (Apt et al. 1996, Falciatore et al. 1999, Zaslavskaia et al. 2000). One seminal discovery was that a modified variant of green fluorescent protein (termed eGFP) from *Aequorea victoria* can be functionally expressed in *P. tricornutum* (wild-type *gfp* and other mutated variants were unsuccessful!). GFP tags have been used extensively in other organisms to follow localization, transport, and turnover of proteins *in vivo* (Sullivan and Kay 1999). Zaslavskaia et al. (2001) have now, for the first time, successfully employed GFP-tagging in a diatom. They transformed *P. tricornutum* with a gene encoding a glut1-eGFP fusion protein. When viewed with the fluorescence microscope, transformants displayed bright fluorescence at the periphery of interphase cells and in the cleavage plane of dividing cells. These features are consistent with membrane localization of the glut1-eGFP fusion protein, which concomitantly was demonstrated by biochemical experiments.

Establishing GFP-tagging technology in diatoms provides a new tool for analyzing cellular biological processes in this group of organisms. One example is the morphogenesis of the ornamented, silicified diatom

cell wall that is formed within a specialized intracellular compartment, termed the silica deposition vesicle (SDV). Based on ultrastructural studies, both actin-filaments and microtubules are believed to be involved in positioning and shaping of the SDV thereby influencing the structure of the forming silica (Pickett-Heaps et al. 1990, Pickett-Heaps 1998). Transforming diatoms with genes encoding GFP-tagged cytoskeletal molecules should allow the dynamics of the diatom cytoskeleton to be monitored by fluorescent microscopy *in vivo* during cell wall formation. Such studies could identify if morphogenesis of certain silica elements does indeed coincide with the formation of characteristic cytoskeletal structures.

The GFP-tagging technique also may be helpful in determining the mechanism that enables chloroplast proteins to enter the complex plastids of diatoms. These organelles are surrounded by a total of four membranes (as compared to only two membranes in higher plants and green and red algae), which requires that diatom chloroplast proteins contain complex targeting information to reach their destination (Apt et al. 1994). Data from *in vitro* studies provide circumstantial evidence that two targeting signals are present within the N-terminal (sequence part) of diatom chloroplast proteins (Bhaya and Grossman 1991, Lang et al. 1998). It should now be possible to test this hypothesis *in vivo* by expressing different versions of N-terminally truncated GFP-tagged chloroplast proteins. Depending on the portion of targeting information that is missing, a truncated fusion protein should then become missorted or "stuck" at a defined stage along the import pathway. Its position would be readily detectable by fluorescence microscopy of live cells. This experimental strategy was employed to analyze protein trafficking to the apicoplast (a remnant chloroplast) in the malarial parasite *Plasmodium falciparum* (Waller et al. 2000).

Apart from the implications for studies of cell biology with diatoms, the results presented by Zaslavskaia et al. (2001) mark an important step toward establishing diatoms as useful organisms for industrial fermentation technology. Large-scale cultivation of obligate photoautotrophic organisms is normally inefficient because light becomes limiting for cell growth even at moderate cell densities. The study of Zaslavskaia et al. (2001) indicates that this problem may be easily solved for diatoms by genetic engineering, which in the future will allow their full potential for biotechnological applications (Apt and Behrens 1999) to be harnessed.

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Apt, K. E. & Behrens, P. W. 1999. Commercial developments in microalgal biotechnology. *J. Phycol.* 35:215–26.

Apt, K. E., Bhaya, D. & Grossman, A. R. 1994. Characterization of genes encoding the light-harvesting proteins in diatoms: biogenesis of the fucoxanthin chlorophyll a/c protein complex. *J. Appl. Phycol.* 6:225–30.

Apt, K. E., Kroth-Pancic, P. G. & Grossman, A. R. 1996. Stable nuclear transformation of the diatom *Phaeodactylum tricornutum*. *Mol. Gen. Genet.* 252:572–9.

Bhaya, D. & Grossman, A. R. 1991. Targeting proteins to diatom plastids involves transport through an endoplasmic reticulum. *Mol. Gen. Genet.* 229:400–4.

Falciatore, A., Casotti, R., Leblanc, C., Abrescia, C. & Bowler, C. 1999. Transformation of nonselectable reporter genes in marine diatoms. *Mar. Biotechnol.* 1:239–51.

Fischer, H., Robl, I., Sumper, M. & Kröger, N. 1999. Targeting and covalent modification of cell wall and membrane proteins heterologously expressed in the diatom *Cylindrotheca fusiformis*. *J. Phycol.* 35:113–20.

Hellebust, J. A. & Lewin, J. 1977. Heterotrophic nutrition. *In* Werner, D. [Ed.] *The Biology of Diatoms*. University of California Press, Berkeley, pp. 169–97.

Lang, M., Apt, K. E. & Kroth, P. G. 1998. Protein transport into "complex" diatom plastids utilizes two different targeting signals. *J. Biol. Chem.* 273:30973–8.

Pickett-Heaps, J., Schmid, A. M. & Edgar, L. 1990. The cell biology of diatom valve formation. *In* Round, F. E. & Chapman, D. J. [Eds.] *Progress in Phycological Research*. Vol. 7. Biopress Ltd., Bristol, pp. 1–186.

Pickett-Heaps, J. 1998. Cell division and morphogenesis of the centric diatom *Chaetoceros decipiens* (Bacillariophyceae) II. Electron microscopy and a new paradigm for tip growth. *J. Phycol.* 34:995–1004.

Smetacek, V. 1998. Diatoms and the silicate factor. *Nature* 391:224–5.

Sullivan, K. F. & Kay, S. A. 1999. Green fluorescent protein. *In* Wilson, L. & Matsudaira, P. [Eds.] *Methods in Cell Biology*. Vol 58. Academic Press, San Diego, pp. 75.

Waller, R. F., Reed, M. B., Cowman, A. F. & McFadden, G. I. 2000. Protein trafficking to the plastid of *Plasmodium falciparum* is via the secretory pathway. *EMBO J.* 19:1794–1802.

Zaslavskaia, L. A., Lippmeier, J. C., Kroth, P., Grossman, A. R. & Apt, K. E. 2000. Transformation of the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) with a variety of selectable marker and reporter genes. *J. Phycol.* 36:379–86.

Zaslavskaia, L. A., Lippmeier, J. C., Shih, C., Ehrhardt, D., Grossmann, A. R. & Apt, K. E. 2001. Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. *Science* 292:2073–5.